Total RNA and Protein Isolation

User manual

NucleoSpin® RNA/Protein

November 2005/Rev. 01

MACHEREY-NAGEL

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1 Kit contents

	NucleoSpin® RNA/ Protein		
	10 preps	**50 preps**	**250 preps**
Cat. No.	**740933.10**	**740933.50**	**740933.250**
Protein Precipitator PP	9 ml	45 ml	225 ml
Protein Loading Buffer PLB (without reducing agent)	2 x 1ml	7.5 ml	5 x 7.5 ml
Reducing agent TCEP	2 x 14 mg	107 mg	5 x 107 mg
Buffer RP1	10 ml	25 ml	125 ml
Buffer RA2	15 ml	15 ml	80 ml
Buffer RA3 (concentrate)*	5 ml	12.5 ml	75 ml
Buffer MDB (Membrane Desalting Buffer)	10 ml	25 ml	125 ml
DNase reaction buffer	3 ml	7 ml	35 ml
DNase I, RNase-free (lyophilized)*	1 vial	1 vial	5 vials
H_2O (RNase-free)	5 ml	15 ml	65 ml
NucleoSpin® Filter units (violet ring)	10	50	250
NucleoSpin® RNA/Protein columns (light blue ring, plus collecting tube)	10	50	250
NucleoSpin® collecting tubes	30	150	750
1.5 ml microcentrifuge tubes	20	100	500
Protocol	1	1	1

* For preparation of working solutions and storage conditions see section 3.

2 Product description

2.1 The basic principle

Introduction

Studies of gene expression at the level of transcription and translation by quantification of RNA and protein are often hampered by the small sample size and the necessity of different – often incompatible – techniques for RNA and protein isolation. Samples may comprise biopsies, tumors, tissues, transgene organisms and others. The NucleoSpin® RNA/Protein Kit however enables isolation of RNA and protein from diverse sample types. Protein and RNA are isolated without splitting the sample prior to protein/RNA extraction. Thus, protein and RNA are obtained from one and the same sample and not from two similar portions of one sample. This is especially valuable for unique, small and precious samples. Isolated RNA is suitable for all common downstream applications. RNA isolated with the NucleoSpin® RNA/Protein Kit is of identical quality as RNA isolated with the well proven NucleoSpin® RNA II Kit. Isolated protein is immediately suitable for SDS-PAGE and Western blot analysis.

RNA and Protein Isolation

One of the most important aspects in the isolation of RNA and Protein is to prevent their degradation during the isolation procedure. With the **NucleoSpin® RNA/Protein** method, cells are lysed by incubation in a solution containing large amounts of chaotropic ions. This lysis buffer immediately inactivates virtually all enzymes (e.g. RNases and proteases) which are present in almost all biological materials. The buffer dissolves even hardly soluble protein, creates appropriate binding conditions which favor adsorption of RNA to the silica membrane and enables protein to pass the specially treated NucleoSpin® RNA/Protein column virtually quantitatively. Expensive and harmful proteinase inhibitors or inhibitor cocktails are not necessary due to the denaturing properties of the lysis buffer. Contaminating DNA, which is also bound to the silica membrane, is removed by a DNase I solution which is directly applied onto the silica membrane during the preparation (RNase-free DNase I is supplied with the kit). Simple washing steps with two different buffers remove salts, metabolites and macromolecular cellular components. Pure RNA is finally eluted under low ionic strength conditions with RNase-free water (supplied).

Protein is isolated from the column flow-through. Protein is precipitated with a special buffer (Protein Precipitator, PP) which effectively precipitates protein. After a washing step the protein pellet is dissolved in Protein Loading Buffer (PLB) containing the odourless reducing agent TCEP. The protein can thus readily be applied to SDS-PAGE analysis.

The RNA and protein preparation using **NucleoSpin® RNA/Protein** kits can be performed at room temperature. The RNA eluate, however, should be treated with care because RNA is very sensitive to trace contaminations of RNases, often found on general lab ware, fingerprints and dust. To ensure RNA stability keep RNA frozen

* For preparation of working solutions and storage conditions see section 3.

at –20°C for short-term or –70°C for long-term storage. Recovered Protein dissolved in Protein Loading Buffer is unproblematic concerning stability.

Simultaneous Isolation of RNA, Protein, and DNA (NucleoSpin® RNA/DNA buffer set)

The NucleoSpin® RNA/DNA buffer set (see ordering information) is a support set for RNA and DNA isolation in conjunction with NucleoSpin® RNA II, NucleoSpin® RNA Plant, or NucleoSpin® RNA/Protein.

This patent pending technology enables successive elution of DNA and RNA from one NucleoSpin® column with low salt buffer and water respectively. DNA and RNA are immediately ready for downstream applications.

The combination with NucleoSpin® RNA/Protein allows parallel isolation of RNA, DNA, and Protein from one undivided sample.

2.2 Kit specifications

- **NucleoSpin® RNA/Protein** kits are recommended for the isolation of total RNA and protein from cultured cells and tissue. The **NucleoSpin® RNA/Protein kits** allow purification of pure RNA with an $A_{260/280}$ ratio generally exceeding 1.9 (measured in TE buffer (pH 7.5)).

- The isolated RNA is ready to use for applications like reverse transcriptase-PCR* (RT-PCR*), primer extension, or RNase protection assays.

- Integrity of RNA, isolated from e.g. eukaryotic cells, is examined by denaturing agarose gel electrophoresis: rRNA bands are sharp, with the 28S band being about twice as intense as the 18S band (see Figure 1).

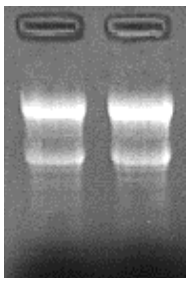


Figure 1: Total RNA from mouse liver was isolated with a NucleoSpin® RNA kit and separated on a 1.2% formaldehyde agarose gel.

- The isolated protein is ready to use for SDS PAGE and Western Blot analysis.

Protein is easily visualized by SDS PAGE:

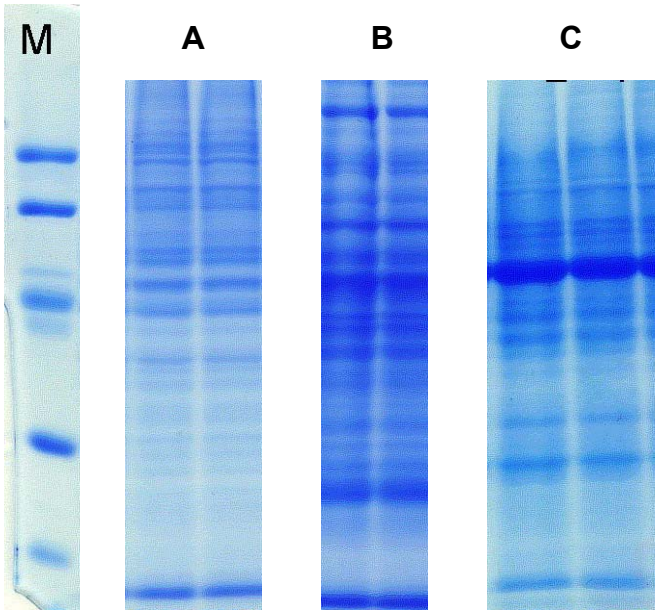


Fig. 2 The protein amount loaded per lane corresponds to 14.000 HeLa cells (A), 0.43 mg liver (B), and1.43 mg garden cress seedling (C), respectively.

Table 1: Kit specifications at a glance	
	NucleoSpin® RNA/Protein
Sample size	up to 5 x 10^6 cells up to 30 mg tissue
RNA yield	up to 70 µg
Protein yield	up to 1200 µg
Elution volume RNA	40 - 120 µl
Resolubilization volume Protein	10 – 100 µl
Binding capacity RNA	100 µg
Time/prep RNA	< 30 min/6 preps
Time/prep protein	35 min/6 preps
Spin column type	mini

- The standard protocol (section 5.1) allows the purification of up to 70 µg of total RNA per **NucleoSpin® RNA/Protein column** from up to 5 x 10^6 cultured cells or 30 mg of tissue (also see table 1). The isolated RNA can be used as template in a RT-PCR*-reaction. Generally, 1 – 10% of the eluate of total RNA prepared from 1 x 10^6 cells or 10 mg of tissue is sufficient as template for RT-PCR. If possible, intron-spanning primers should be used for RT-PCR.

- The RNA prepared from such high amounts is generally free of residual DNA, although minute traces of DNA may remain in the preparation, if large amounts of material rich in nucleic acids are used. However, if the isolated RNA will be used as template in a RT-PCR*-reaction, we recommend that lower quantities of sample be used, depending on cell or tissue type, in the range of 1 x 10^6 cultured cells or 10 mg of tissue resulting in about 20 µg of RNA.

- The kit can be used for preparing RNA from different amounts of sample material according to the following table:

Sample	Amount
Cultured animal cells (e.g. HeLa cells)	up to 5 x 10^6
Animal tissue	up to 30 mg
Bacteria	up to 1 x 10^9
Yeast	up to 5 x 10^7

- Depending on sample type, the average yield is around 5 µg -70 µg total RNA (see Table 2). The $A_{260/280}$ ratio, indicating purity of the RNA, generally exceeds 1.9.

Table 2: Overview on average yields of total RNA isolation using NucleoSpin® RNA/Protein

Sample	**average yield (µg)**
8 x 10^4 HeLa cells	1.5
4 x 10^5 HeLa cells	4
1 x 10^6 HeLa cells	14
2 x 10^6 HeLa cells	21
2.5 x 10^6 HeLa cells	25
5 x 10^6 HeLa cells	50

Protein yield

Protein yield depends on sample type, amount and quality as well as on homogenization efficiency. Further, the utilized quantification method influences determined protein yield. The following values were determined according to the method described in appendix section 6.1 and shall serve as a guideline for expected protein yield. It is assumed, that the complete sample amount is processed, i.e. the complete lysed sample is – after ethanol addition – loaded onto the column and the complete 700 µl flow through is subjected to protein precipitation. In many cases precipitation of only a portion of the column flow through (e.g. 100 µl) is recommended and will yield enough protein in terms of absolute amount and concentration for SDS-PAGE / Western analysis.

Sample type and amount	Protein yield
cultured human cells, e.g. HeLa, approx. 10^6 cells	~ 50 – 150 µg
plants, e.g. garden cress, approx. 100 mg	~ 150 – 350 µg
animal tissue, e.g. pig liver, approx. 30 mg	~ 500 – 1200 µg

2.3 Handling, preparation, and storage of starting materials

RNA is not protected against digestion until the sample material is flash frozen or disrupted in the presence of RNase inhibiting or denaturing agents. Therefore it is important that samples are flash frozen in liquid N_2 immediately and stored at –70°C or processed as soon as possible. Samples can be stored in lysis buffer RP1 after disruption at –70°C for up to one year, at +4°C for up to 24 hours or up to several hours at room temperature. Frozen samples are stable up to 6 months. Frozen samples in buffer RP1 should be thawed slowly before starting with the isolation of total RNA.

Wear gloves at all times during the preparation. Change gloves frequently.

Cultured animal cells are collected by centrifugation and directly lysed by adding buffer RP1 according to step 2 of the standard protocol (see section 5.1).

Cell lysis of adherent growing cells in a culture dish:

Completely aspirate cell-culture medium, and continue immediately with the addition of lysis buffer RP1 to the cell-culture dish. Avoid incomplete removal of the cell-culture medium in order to allow full lysis activity of the lysis buffer.

To trypsinize adherent growing cells:

Aspirate cell-culture medium and add and equal amount of PBS in order to wash the cells. Aspirate PBS. Add 0.1 – 0.3% trypsin in PBS and incubate for a appropriate time to detach the cells from the dish surface. After cell detachment, add medium, transfer cells to an appropriate tube (not supplied), and pellet by centrifugation for 5 min at 300 x g. Remove supernatant and continue with the addition of lysis buffer to the cell pellet.

Animal tissues are often solid and must therefore be broken up mechanically as well as lysed. Depending on the disruption method, the viscosity of the lysed sample has to be reduced further for optimal results. It is essential for efficient RNA preparation that all the RNA contained in the sample is released from the cells by disruption and that the viscosity of the sample is reduced by homogenization.

The most commonly used technique for disruption of animal tissues is grinding with a **pestle and mortar.** Grind the sample to a fine powder in the presence of liquid N_2. Take care that the sample does not thaw during or after grinding or weighing and add the frozen powder to an appropriate aliquot of buffer RP1 containing β-mercaptoethanol and mix immediately. The broken-up tissue must then be homogenized with a **NucleoSpin® Filter/ Filter L** unit or by passing ≥ 5 through a 0.9 mm syringe needle.

Thawing of undisrupted animal tissue should be exclusively done in the presence of buffer RP1 during simultaneous mechanical disruption, e.g. with a **rotor-stator homogenizer**. This ensures that the RNA is not degraded by RNases before the

preparation has started. The spinning rotor disrupts and simultaneously homogenizes the sample by mechanical shearing of DNA within seconds up to minutes (homogenization time depends on sample). Take care to keep the rotor tip submerged in order to avoid excess foaming. To degenerate evolved foam, centrifuge 1 min at 400 x g. Select a suitably sized homogenizer (5-7 mm diameter rotors can be used for homogenization in microcentrifuge tubes).

Bacteria and yeasts have to be incubated in lysozyme or lyticase/zymolase solutions, respectively (see support protocols in section 4). By this treatment, the robust cell walls of these organisms are digested or at least weakened, which is essential for effective cell lysis by buffer RP1. For microorganisms with extremely resistant cell walls – like some Gram-positive bacterial strains – it may be necessary to optimize the conditions of the treatment with lytic enzymes or the cultivation conditions. After lysis, homogenization is achieved by the use of a **NucleoSpin® Filter** units or the syringe-needle method.

2.4 Guideline for appropriate sample amount, precipitation, and resolubilization volume for protein isolation

The following table shall serve as a first guide for choosing appropriate amounts of sample material, precipitation, and resolubilization volume. Depending on sample type and downstream application (Coomassie or silver stain, sensitivity of antibody, detection system) appropriate volumes might deviate from the table below and have to be determined experimentally.

amount of	cultivated cells (e.g. HeLa)			animal tissue (e.g. liver)			plant tissue (e.g. garden cress leaf)		
sample	10^6	10^5	10^4	30 mg	3 mg	0.3mg	100mg	10 mg	1 mg
lysis buffer RP1	350 µl								
ethanol	350 µl								
column flow through to be precipitated	35 µl	350 µl	700 µl	35 µl	350 µl	700 µl	35 µl	350 µl	700 µl
sample buffer PLB used for protein pellet solubilisation	100 µl	100 µl	20 µl	100 µl	100 µl	20 µl	100 µl	100 µl	20 µl
protein sample to be analysed on SDS PAGE with coomassie stain	10 µl								
protein sample to be analysed on SDS PAGE with silver stain	1 µl								
protein sample analysed on western blot	1 – 10 µl								

2.5 Elution procedures for RNA

It is possible to adapt elution method and volume of water used for the subsequent application of interest. In addition to the standard method described in the individual protocols (recovery rate about 70-90%) there are several modifications possible.

- **High yield:** Perform two elution steps with the volume indicated in the individual protocol. About 90-100% of bound nucleic acid will be eluted.

- **High yield and high concentration:** Elute with the standard elution volume and apply the eluate once more onto the column for reelution.

Eluted RNA should immediately be put and always kept on ice for optimal stability because almost omnipresent RNases (general lab ware, fingerprints, dust) will degrade RNA. For short-term storage freeze at –20°C, for long-term storage freeze at –70°C.

3 Storage conditions and preparation of working solutions

Attention:

Buffers RP1, RA2, and MDB contain guanidine thiocyanate. Wear gloves and goggles!

- Store lyophilized **RNase-free DNase I** at +4°C on arrival (stable up to 1 year).
- Store lyophilized **reducing agent TCEP** at +4°C on arrival.
- All other kit components should be stored at room temperature (20-25°C) and are stable up to one year. Storage at lower temperatures may cause precipitation of salts.
- Check that 70% ethanol is available as additional solution to adjust binding conditions in the RP1-lysate.
- Check that 50% ethanol is available as additional solution to wash the protein pellet.

Before starting any **NucleoSpin® RNA/Protein** protocol prepare and consider the following:

- **RNase-free DNase I**: Add indicated volume of RNase-free water (see table below) to the DNase I vial and incubate for 1 min at room temperature. Gently swirl the vials to completely dissolve the DNase. Be careful not to mix DNase I vigorously as DNase I is sensitive to mechanical agitation. Dispense into aliquots and store at –18°C. The frozen working solution is stable for 6 months. Do not freeze/thaw the aliquots more than three times.
- **Buffer RA3**: Add the indicated volume of 96 – 100% ethanol (see table below) to the RA3 concentrate. Store buffer RA3 at room temperature (20-25°C) for up to one year.
- **Protein Loading Buffer PLB and reducing agent TCEP**: For SDS-PAGE under reducing conditions transfer PLB (without reducing agent) to the lyophilized reducing agent TCEP. Mix well until the reducing agent is dissolved completely (this process will require several minutes)*. Protein Loading Buffer containing reducing agent TCEP (PLB-TCEP) is stable for several days at room temperature (18 – 25°C) and several month at 4°C. For long term storage of PLB-TCEP keep at -20°C.

* For 50 and 250 prep kits: For better handling PLB-TCEP may be transferred into the original PLB vial (with screw cap).

- If SDS-PAGE at non-reducing conditions is intended consider the following: A: Omit addition the reducing agent TCEP to buffer PLB. B: Omit addition of β-mercaptoethanol to lysis buffer RP1.

- If other reducing agents than TCEP are preferred (e.g. DTT, β-mercaptoethanol), appropriate amounts should be added to PLB. Please consider limited stability of DTT compared to TCEP.

	NucleoSpin® RNA/Protein		
	10 preps	**50 preps**	**250 preps**
Cat. No.	**740933.10**	**740933.50**	**740933.250**
Buffer RA3 (concentrate)	5 ml add 20 ml ethanol	12.5 ml add 50 ml ethanol	3 x 25 ml add to each vial 100 ml ethanol
DNase I, RNase-free (lyophilized)	1 vial add 230 µl RNase-free water	1 vial add 540 µl RNase-free water	5 vials add to each vial 540 µl RNase-free water

4 Safety instructions – risk and safety phrases

The following components of the NucleoSpin® RNA/Protein kits contain hazardous contents.

Wear gloves and goggles and follow the safety instructions given in this section.

Component	Hazard Contents	Hazard Symbol		Risk Phrases	Safety Phrases
DNase I	DNase I, lyophilized	Xn*	May cause sensitization by inhalation and skin contact	R 42/43	S 22-24
Buffer RP1	guanidine thiocyanate	Xn*	Harmful by inhalation, in contact with skin and if swallowed	R 20/21/22	S 13
Buffer RA2	guanidine thiocyanate	Xn*	Harmful by inhalation, in contact with skin and if swallowed	R 20/21/22	S 13
MDB	guanidine thiocyanate < 10%	Substance does not have to be specially labeled as hazardous			
Reducing agent TCEP	Tris (2-carboxyethyl) phosphine Hydrochloride	Xi*	Causes burns.	R 34	S 26-27-36/37/39

Risk Phrases

R 20/21/22 Harmful by inhalation, in contact with the skin and if swallowed

R 34 Causes burns

R 42/43 May cause sensitization by inhalation and skin contact

Safety Phrases

S 13 Keep away from food, drink and animal feedstuffs

S 22 Do not breathe dust

S 24 Avoid contact with the skin

S 26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice

S 27 Take off immediately all contaminated clothing

S 36/37/39 Wear suitable protective clothing, gloves and eye/face protection

* Label not necessary, if quantity below 125 g or ml (concerning 67/548/EEC Art. 25, 1999/45/EC Art. 12 and German GefStoffV § 42 and TRGS 200 7.1)

5 Protocols

5.1 Total RNA and protein purification from cultured cells and tissue with NucleoSpin® RNA/Protein

Joint protocol steps for RNA and protein purification.

1 Homogenization of sample

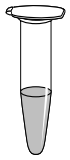
Disrupt up to **30 mg of tissue** (for homogenization methods see section 2.4)

disrupt sample

Up to **5 x 10^6** eukaryotic **cultured cells** are collected by centrifugation and lysed by addition of RP1 directly

2 Cell lysis

Add **350 µl buffer RP1** and **3.5 µl β-mercaptoethanol** to the cell pellet or to ground tissue and vortex vigorously.

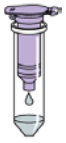


+ 350 µl RP1
+ 3.5 µl β-me

3 Filtration of the lysate

Reduce viscosity and clear the lysate by **filtration** through **NucleoSpin® Filter units**: Place NucleoSpin® Filter units (violet) in a collecting tube, apply the mixture, and **centrifuge for 1 min at 11,000 x g.**

The lysate may be passed alternatively ≥ 5 times through a 0.9 mm needle (20 gauge) fitted to a syringe.



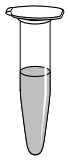
1 min
11,000 x g

In case of visible pellet formation (depending on sample amount and nature) transfer supernatant without any formed pellet to a new 2 ml centrifuge tube (not included).

Important: To process higher amounts of cells (>1 × 10^6) or tissue (>10 mg), the lysate should first be homogenized using the 0.9 mm needle (20 gauge), followed by filtration through NucleoSpin® filter units.

4 Adjust RNA binding conditions

Discard the NucleoSpin® Filter unit and **add 350 µl ethanol (70%)** to the homogenized lysate and mix by pipetting up and down (approx. 5 times).



+ 350 µl 70% EtOH

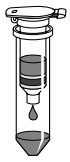
mix

After addition of ethanol a stringy precipitate may become visible which will not affect the RNA isolation. Be sure to disaggregate any precipitate by mixing and to load all of the disaggregated precipitate on the column as described in step 5. Do not centrifuge at this stage in order to avoid sedimentation of any precipitate.

5 Bind RNA

For each preparation, take one **NucleoSpin® RNA/Protein column** (light blue) placed in a 2 ml centrifuge tube and **load** the lysate. Centrifuge **for 30 s at 11,000 × *g***. Place the column in a new collecting tube.

RNA and DNA are bond to the column matrix, protein is contained in the flow-through.



load lysate

Maximal loading capacity of NucleoSpin® RNA/Protein columns is 750 µl. Repeat the procedure if larger volumes are to be processed.

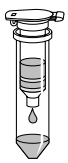
30 s 11,000 × *g*

For **RNA** isolation continue with step 6.
It is recommended first to continue RNA isolation protocol and do protein purification subsequently.

For **protein** isolation recover flow-through and continue with step 10.
The protein containing flow-through is stable for several hours at 4-8°C.

Further steps for RNA purification (steps 6 – 9)

6 Desalt silica membrane



+ 350 µl MDB

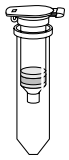
Add 350 µl MDB (Membrane Desalting Buffer) and centrifuge at **11,000 x g** for **1 min** to dry the membrane.

1 min 11,000 x *g*

Salt removal will make the following DNase I digest much more effective. If the column outlet has come into contact with the flow-through for any reason, discard the flow-through and centrifuge again for 30 s at 11,000 x g.

7 Digest DNA

Prepare DNase reaction mixture in a sterile microcentrifuge tube: for each isolation, add **10 µl** reconstituted **DNase I** (also see section 3) to **90 µl DNase reaction buffer**. Mix by flicking the tube.

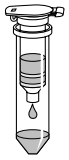


+ 95 µl DNase reaction mixture

Apply 95 µl DNase reaction mixture directly onto the center of the silica membrane of the column. Incubate at **room temperature** for **15 min**.

RT
15 min

8 Wash and Dry silica membrane



+ 200 µl RA2

1st wash

Add 200 µl buffer RA2 to the NucleoSpin® RNA/Protein column. Centrifuge for **30 s** at **11,000** × ***g***. Place the column into a new collecting tube.

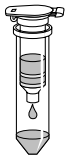
30 s
11,000 × ***g***

Buffer RA2 will inactivate the DNase.

2nd wash

Add 600 µl buffer RA3 to the NucleoSpin® RNA/Protein column. Centrifuge for **30 s** at **11,000 x g**. Discard flow-through and place the column back into the collecting tube.

+ 600 µl RA3



30 s
11,000 × ***g***

3rd wash

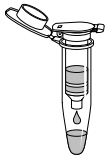
Add 250 µl buffer RA3 to the NucleoSpin® RNA/PROTEIN column. Centrifuge for **2 min** at **11,000 x g** to dry the membrane completely. Place the column into a nuclease-free 1.5 ml microcentrifuge tube (supplied).

+ 250 µl RA3

2 min
11,000 x g

If for any reason, the liquid level in the collecting tube has reached the NucleoSpin® RNA/Protein column after centrifugation, discard flow-through and centrifuge again.

9 Elute highly pure RNA



+ 60 µl H_2O (RNase-free)

Elute the RNA in **60 µl H_2O (RNase-free)** (supplied) and centrifuge at **11,000 x g** for **1 min**.

If higher RNA concentrations are desired, elution can be done with 40 µl for the NucleoSpin® RNA/Protein kit. Overall yield, however, will decrease when using smaller volumes.

For further alternative elution procedures see section 2.5.

1 min
11,000 x g

Further steps for protein purification (steps 10 – 13)

Perform sample homogenization, cell lysis, lysate filtration, adjusting of nucleic acid binding condition, and binding of nucleic acids to the NucleoSpin RNA binding column according to the NucleoSpin® RNA/Protein kit standard protocol, steps 1-5.

Use the NucleoSpin RNA/Protein column flow-through (i.e. the ethanolic lysate which has been passed throught the RNA binding column and is as such deprived of nucleic acids) as starting point for protein precipitation.

10 Protein precipitation

Transfer an appropriate amount (10 – 700 µl) of flow-though into a fresh 1.5 ml microcentrifuge tube (supplied).
See section 2.4 as guideline for choosing an appropriate amount.

Add **one volume** of **PP** (Protein Precipitator). Mix vigorously. Incubate mixture at room temperature for approximately 10 minutes.

Note: For samples of moderate to high protein content (e.g. 100 mg young plant leaf, 30 mg liver) this incubation step may be omitted. For samples of low to medium protein content (e.g. 15 mg young plant leaf) the 10 min incubation increases protein yield relative to no incubation significantly). An incubation of longer than one hour does not further increase protein yield.

Centrifuge for 5 min at 11.000 x g.

11 Protein wash

Remove supernatant by pipetting or decanting as complete as possible.

Add **500 µl** of **50% ethanol** to the pellet (mixing or incubation at this step is not necessary).

Centrifuge 1 min at 11,000 x g.

Remove supernatant by pipetting or decanting as completely as possible.

Note: Protein precipitate at this stage is quite different in appearance depending on kind and amount of starting material. The appearance might be: no visible pellet or precipitate (e.g. for 10.000 cell, 0.3 mg liver and 1 mg leaf samples); a greenish tube wall coating on one side of the tube (for e.g. leaf material); green or white pellet at the bottom of the tube (e.g. for leaf and liver samples, respectively); green or white crumbs at one side of the inner wall of the centrifuge tube (e.g. for leaf and liver samples, respectively). If no precipitate is visible, mark the side of the tube where a precipitate is expected in order to avoid touching this side of the inner tube wall with the pipet tip during the washing step.

12 Dry protein pellet

Dry precipitate for **5 – 10 min** at **room temperature**; keep lid open.

Note: Large pellets (e.g. complete precipitation of 700 µl column flow-through form a 30 mg liver sample) need longer drying duration. Samples which are dried incomplete my cause problems when loading the sample onto the gel due to residual ethanol content. No problems with over-drying have been observed with small-sized pellets.

13 Protein sample preparation

Add **20 – 100 µl PLB** (Protein Loading Buffer). See section 2.4 as guideline for choosing an appropriate amount.

Disaggregate large and visible pellets with a pipet tip to facilitate subsequent protein dissolution; this is not necessary for small and invisible pellets.

Incubate for **3 min at 95 – 98°C** for complete protein dissolving and denaturation.

Let sample cool down to room temperature.

Centrifuge for 1 min at 11,000 x g to pellet residual insolvable material.

Note: Depending on sample amount and nature there might be no visible pellet of insolvable material up to large pellets of different size and structure. Do not disturb precipitates at this stage. Protein will be in the supernatant. Do not centrifuge samples cooled down to 0 – 4°C. SDS may precipitate at this temperature.

Recover supernatant for further analysis.

Note: At this stage samples can be stored at -20°C / 4°C for several month / days. After storage, equilibrate sample to room temperature, mix and then centrifuge briefly before withdrawal of sample aliquots. Repeated sample denaturing for 3 min at 95 – 98°C is not necessary. Repetitive withdrawal, freezing and thawing for at least three times has shown constant sample quality.

5.2 Support protocol NucleoSpin® RNA/Protein: Total RNA preparation from biological fluids (e.g. serum, culture medium)

1 Homogenization of sample

Not necessary!

2 Cell lysis

Add 350 µl buffer RP1 to 100 µl of sample and vortex vigorously.

3 Filtration of the lysate

Not necessary!

4 Adjust RNA binding conditions

Add **350 µl** of **ethanol (70%)** to the lysate and mix by pipetting up and down (approx. 5 times).

Proceed with step 5 of the NucleoSpin® RNA/Protein standard protocol (section 5.1)

5.3 Support protocol NucleoSpin® RNA/Protein: Total RNA preparation from up to 10^9 bacterial cells

1 Homogenization of sample

Resuspend the **bacterial cell pellet** (Gram-negative strains) in **100 µl TE buffer** (10 mM Tris-HCl, 1 mM EDTA; pH 8) containing **0.2 mg/ml lysozyme** by vigorous vortexing. Incubate at **37°C** for **10 min**.

For preparation of RNA from Gram-positive bacteria, resuspend cells in 100 µl TE containing 2 mg/ml lysozyme. It may be necessary to optimize incubation time and lysozyme concentration, depending on the bacterial strain.

2 Cell lysis

Add 350 µl buffer RP1 and **3.5 µl β-mercaptoethanol** to the suspension and vortex vigorously.

3 Filtration of lysate

Reduce viscosity and turbidity of the solution by filtration through **NucleoSpin® Filter units**. Place NucleoSpin® Filter units in collecting tubes, apply mixture, and centrifuge for **1 min** at **11,000 x g**.

In case of visible pellet formation (depending on sample amount and nature) transfer supernatant without any formed pellet to a new 2 ml centrifuge tube (not included).

Alternatively, the lysate may be passed ≥ 5 times through a 0.9 mm needle (20 gauge) fitted to a syringe.

4 Adjust RNA binding conditions

Add 350 µl of **ethanol (70%)** and mix by pipetting up and down (approx. 5 times). Proceed with step 5 of the NucleoSpin® RNA/Protein standard protocol (section 5.1).

Because of the much greater concentration of genome equivalents in a nucleic acid preparation of bacteria compared with eukaryotic material, it may be necessary to use a lower quantity of cells for the preparation.

5.4 Support protocol NucleoSpin® RNA/Protein: Total RNA preparation from up to 5 x 10^7 yeast cells

1 Homogenization of sample

Harvest 2-5 ml of **YPD culture (5,000 × *g*; 10 min)**. Resuspend pellet in **sorbitol/lyticase buffer** (50-100 U lyticase or zymolase in 1 M sorbitol/100 mM EDTA) and incubate at **30°C** for **30 min**. Pellet the resulting spheroplasts by centrifugation **(1,000 × *g*; 10 min)**.

It may be necessary to optimize incubation time and lyticase/zymolase concentration, depending on the yeast strain.

2 Cell lysis

Add 350 µl buffer RP1 and **3.5 µl β-mercaptoethanol** to the suspension and vortex vigorously.

3 Filtration of lysate

Reduce viscosity and turbidity of the solution by filtration through **NucleoSpin® Filter units**. Place NucleoSpin® Filter units in collecting tubes, apply mixture, and centrifuge for **1 min** at **11,000 x g**.

In case of visible pellet formation (depending on sample amount and nature) transfer supernatant without any formed pellet to a new 2 ml centrifuge tube (not included).

Alternatively, the lysate may be passed ≥ 5 times through a 0.9 mm needle (20 gauge) fitted to a syringe.

Proceed with step 4 of the NucleoSpin® RNA/Protein standard protocol (section 5.1)

Due to the much higher concentration of genome equivalents in a nucleic acid preparation of yeasts compared with cultured cells or tissue material, it may be necessary to use a lower quantity of cells for the preparation.

6 Appendix

6.1 Quantification of protein in sample buffer

Quantification of protein in the sample buffer is occasionally helpful prior to SDS PAGE and Western Blot analysis. However, major protein quantification assays are influenced by/incompatible with SDS/reducing agents commonly present in protein sample buffers for SDS PAGE.

The procedure presented below (based on the publication of Karlsson *et al*, 1994), is suitable for quantification of protein in Protein Loading Buffer PLB.

Nucleic acids disturb protein quantification as described by Karlsson. Protein samples obtained with the NucleoSpin® RNA/Protein kit are virtually free of nucleic acids, thus, protein quantification is not affected.

Upon addition of TCA (Trichloracetic acid) to the sample, protein precipitates and causes turbidity. The degree of turbidity is used for quantification relative to a sample with known protein concentration. This test enables determination of protein concentration in the range 5 ng/µl – 20 µg/µl by using variable sample volumes of 1 – 60 µl.

Recommended sample volume (Protein dissolved in PLB-TCEP)	For protein concentration in the range of
60 µl	0.005 – 0.33 µg/µl
20 µl	0.015 – 1.0 µg/µl
1 µl	0.3 – 20 µg/µl

Material

TCA 60% (Trichloracetic acid, not supplied)

Protein Loading Buffer with reducing agent (PLB-TCEP)

BSA (Bovine Serum Albumin, not supplied)

Multititer plate (not supplied)

Composition of PLB-TCEP

125 mM BisTris (Bis-(2-hydroxyehtyl)-imino-tris(hydroxymethyl)-methane)
10% SDS (sodium dodecyl sulphate)
50 mM TCEP (Tris(2-carboxyethyl)phosphine Hydrochloride)
20% glycerol
0.02% brome phenol blue
pH 6.8

Method

Prepare a BSA stock solution with 40 mg/ml BSA in H_2O.

Prepare a BSA dilution series:

	BSA solution	PLB-TCEP	resulting BSA concentration	BSA in 20 µl
#1	2.5 µl BSA stock solution (40 mg/ml)	97.5 µl	1 µg/µl	20 µg
#2	50 µl from #1	50 µl	0.5 µg/µl	10 µg
#3	50 µl from #2	50 µl	0.25 µg/µl	5 µg
#4	50 µl from #3	50 µl	0.125 µg/µl	2.5 µg
#5	50 µl from #4	50 µl	0.063 µg/µl	1.25 µg
#6	50 µl from #5	50 µl	0.031 µg/µl	0.625 µg
#7	50 µl from #6	50 µl	0.016 µg/µl	0.312 µg
#8	-	50 µl	0 µg/µl	0 µg

The prepared BSA dilution series is sufficient for subsequent determination of two calibration curves.

1 Add 20 µl of each dilution series sample #1-8 in microtiter plates wells.

2 Add 20 µl of samples (protein dissolved in PLB-TCEP) with unknown protein concentration to further wells (alternatively 1 – 60 µl).

3 Add 40 µl PLB-TCEP to each well. Final volume: 60 µl (alternatively add 0– 55 µl if other volumes than 20 µl of sample are used in step 2).

4 Add 40 µl TCA (60%) to each well.

5 Mix until complete colour change from blue to yellow.

6 Incubate for 30 min (±3 min) at room temperature.

7 Measure absorbance at 570 nm*.

8 Determine protein concentration of samples in relation to dilution series.

* Measurement of absorption in the range of 530-700 nm is suitable and will typically result in correlation coefficients of ≥ 0.99 (concentration of BSA dilution series vs. obtained absorption values).

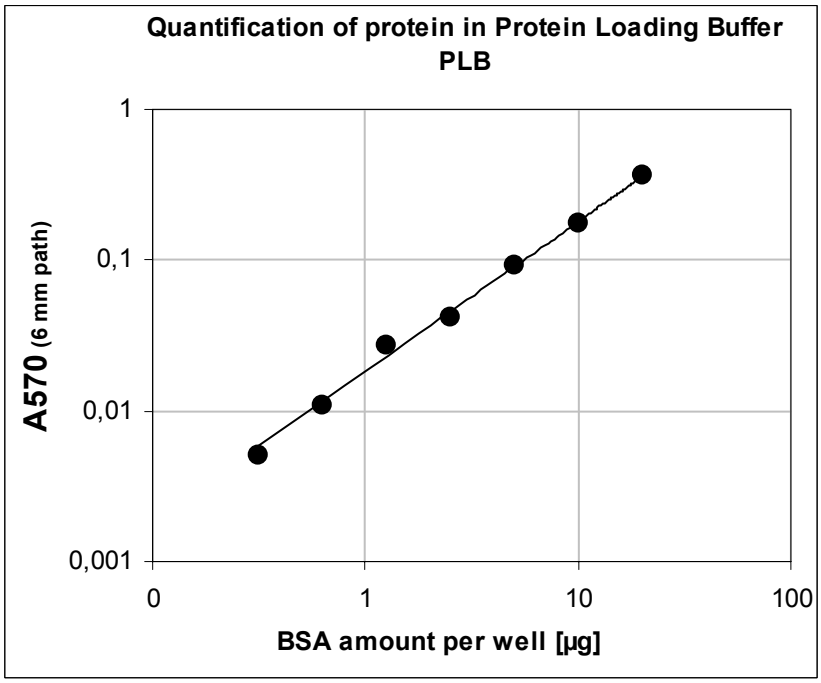


Fig.3 BSA standard curve for determination of protein in Protein Loading Buffer PLB.

6.2 Troubleshooting

Problem	Possible cause and suggestions
RNA is degraded/ no RNA obtained	*RNase contamination* • Create an RNase free working environment. Wear gloves during all steps of the procedure. Change gloves frequently. Use of sterile, disposable polypropylene tubes is recommended. Keep tubes closed whenever possible during the preparation. Glassware should be oven-baked for at least 2 hours at 250°C before use.
Poor RNA quality or yield	*Reagents not applied or restored properly* • Reagents not properly restored. Add the indicated volume of nuclease-free water to DNase I vial and 96% ethanol to buffer concentrate RA3 and mix. Reconstitute and store lyophilized DNase I according to instructions given in section 3. • Sample and reagents have not been mixed completely. Always vortex vigorously after each reagent has been added. • No ethanol has been added after lysis. Binding of RNA to the silica membrane is only effective in the presence of ethanol. *Kit storage* • Reconstitute and store lyophilized DNase I according to instructions given in section 3. • Store other kit components at room temperature. Storage at low temperatures may cause salt precipitation. • Keep bottles tightly closed in order to prevent evaporation or contamination.

Problem	Possible cause and suggestions
Poor RNA quality or yield *continued*	*Ionic strength and pH influence A_{260} absorption as well as ratio $A_{260/280}$* • For adsorption measurement, use 5 mM Tris pH 8.5 as diluent. Please see also: - Manchester, K L. 1995. Value of A260/A280 ratios for measurement of purity of nucleic acids. Biotechniques 19, 208-209. - Wilfinger, W W, Mackey, K and Chomczyski, P. 1997. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. Biotechniques 22, 474-481. *Sample material* • Sample material not stored properly. Whenever possible, use fresh material. If this is not possible, flash freeze the samples in liquid N_2. Samples should always be kept at –70°C. Never allow tissues to thaw before addition of buffer RP1. Perform disruption of samples in liquid N_2. • Insufficient disruption and/or homogenization of starting material. Ensure thorough sample disruption and use NucleoSpin® Filter/ Filter L units for easy homogenization of disrupted starting material.
Clogged NucleoSpin® column/Poor RNA quality or yield	*Sample material* • Too much starting material used. Overloading may lead to decreased overall yield. Reduce amount of sample material or use larger volume of RP1. • Insufficient disruption and/or homogenization of starting material. Ensure thorough sample disruption and use NucleoSpin® Filter/ Filter L units for easy homogenization of disrupted starting material.

Problem	Possible cause and suggestions
Contamination of RNA with genomic DNA	*DNase I not active* • Reconstitute and store lyophilized DNase I according to instructions given in section 3. *DNase solution not properly applied* • Pipette DNase I solution directly onto the center of the silica membrane. *Too much cell material used* • Reduce quantity of cells or tissue used. *DNA detection system too sensitive* • The amount of DNA contamination is significantly reduced during the on-column DNase I digestion. Anyhow we can not guarantee that the purified RNA is 100% free of DNA, therefore in very sensitive applications it might be possible to detect DNA. The NucleoSpin® RNA Protein/II/Plant system is checked by the following procedure: One million HeLa cells are subjected to RNA isolation according to the protocol. RNA eluate is used as template for PCR detection of a 1kb fragment in a 30 cycle reaction. Generally, no PCR fragment is obtained if the DNase is applied, however, a strong PCR fragment is obtained if DNase is omitted. The eventuality of DNA detection with PCR increases with: - the number of DNA copies per preparation: single copy target < plastidial/ mitochondrial target < plasmid transfected into cells - decreasing of PCR amplicon size • Use larger PCR targets (e.g. >500bp) or intron spanning primers if possible.

Problem	Possible cause and suggestions
Suboptimal performance of RNA in downstream experiments	*Carryover of ethanol or salt* • Do not let the flow-through touch the column outlet after the second RA3 wash. Be sure to centrifuge at the corresponding speed for the respective time in order to remove ethanolic buffer RA3 completely. • Check if buffer RA3 has been equilibrated to room temperature before use. Washing at lower temperatures lowers efficiency of salt removal by RA3. *Store isolated RNA properly* • Eluted RNA should always be kept on ice for optimal stability since trace contaminations of omnipresent RNases (general lab ware, fingerprints, dust) will degrade the isolated RNA. For short term storage freeze at –20°C, for long term storage freeze at –70°C.
trouble with resolubilization of precipitated protein in PLB-TCEP	*Protein pellets exceeding several millimeters in size are hard to dissolve.* • Use smaller volumes of column flow-through for protein precipitation in order to obtain small sized pellets. Even invisible protein pellets commonly yield enough protein for SDS PAGE and Western Blot analysis
Protein dissolved in PLB-TCEP escapes SDS PAGE gel slot immediately after loading	*Protein pellet has not been dried sufficiently and contains residual ethanol.* • Increase drying time or decrease pellet size by precipitating a smaller volume of column flow-through
Unclear results with commonly used protein quantification systems	*Most commonly used protein quantification systems are incompatible with concentrations of SDS and/or reducing agents present in Protein Loading Buffer.* • Use quantification method as described in section 6.1, which is compatible with Protein Loading Buffer PLB

Problem	Possible cause and suggestions
No protein precipitate (pellet) visible	*A small sample amount was used and/or a small volume of column flow-through was used for precipitation.* • Formation of a visible protein pellet is not required for sufficient protein recovery. Even invisible protein pellets commonly yield enough protein for SDS PAGE and Western Blot analysis

6.3 Literature

Coombs LM, Pigott D, Proctor A, Eydmann M, Denner J, and Knowles MA (1990): Simultaneous isolation of DNA, RNA, and antigenic protein exhibiting kinase activity from small tumor samples using guanidine isothiodyanate. Analytical Biochemistry 188, pp338-343.

Banerjee S, Smallwood A, Chambers AE, and Nicolaides K (2003) : Quantitative recovery of immunoreactive proteins from clinical samples following RNA and DNA isolation. BioTechniques 35 (3), pp 450-456.

Getz EB, Xiao M, Chakrabarty T, Cooke R and Selvin PR (1999): A comparison between the sulfhydryl reductants Tris(2-carboxyethyl)phosphine and Dithiothreitol for use in protein biochemistry. Analytical Biochemistry 273, 73-80.

Hoemann CD, Sun J, Chrzanowski V, and Buschmann MD (2002): A multivalent assay to detect glycosaminoglycan, protein, collagen, RNA, and DNA content in milligam samples of cartilage or hydrogel-based repair cartilage. Analytical Biochemistry 300, 1-10.

Karlsson JO, Ostwald K, Kabjörn C, and Andersson M (1994): A method for protein assay in Laemmli buffer. Analytical Biochemistry 219, 144-146.

6.4 Ordering information

Product	Cat. No.	Pack of
NucleoSpin® RNA II	740955.10	10
NucleoSpin® RNA II	740955.20	20
NucleoSpin® RNA II	740955.50	50
NucleoSpin® RNA II	740955.250	250
NucleoSpin® RNA L	740962.20	20
NucleoSpin® RNA Clean-up	740948.10	10
NucleoSpin® RNA Clean-up	740948.50	50
NucleoSpin® RNA Clean-up	740948.250	250
NucleoSpin® RNA/DNA buffer set	740944	100
DNase I set	740963	1 set
NucleoSpin® Filter	740606	50
NucleoSpin® collection tubes	740600	1000
Porablot	see price list	
Blotting paper	see price list	

6.5 Product use restriction / warranty

NucleoSpin® RNA/Protein kits components were developed, designed and sold **for research purposes only**. They are suitable ***for in vitro uses only***. No claim or representation is intended for its use to identify any specific organism or for clinical use (diagnostic, prognostic, therapeutic, or blood banking).

It is rather the responsibility of the user to verify the use of the **NucleoSpin® RNA/Protein** kits for a specific application range as the performance characteristic of this kit has not been verified to a specific organism.

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